Proposal for C-Hordein as Reference Material in Gluten Quantification

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Contaminant barley

Pure oat

Gluten quantification: Gluten-free?

R5 sandwich ELISA

Overestimation When using gliadin as reference material

True gluten content

Correct content when using C-hordein as reference material

Proposal for C-hordein as reference material in gluten quantification

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Abstract

The concentration of residual barley prolamin (hordein) in gluten-free products is overestimated by the R5 ELISA method when calibrated against the wheat gliadin standard. The reason for this may be that the composition of the gliadin standard is different from the composition of hordeins. This study showed that the recognition of whole hordein by R5 antibody mainly came from C-hordein, which is more reactive than the other hordeins. The proportion of C-hordein in total hordein ranged from 16% to 33% of common Finnish barley cultivars used in this study, and was always higher than that of omega-gliadin, the homologous protein class in the gliadin standard, which may account for the overestimation. Thus, a hordein standard is needed for barley prolamin quantification instead of the gliadin standard. When gluten-free oat flour was spiked with barley flour, the prolamin concentration was overestimated 1.8-2.5 times with the gliadin standard, whereas estimates in the correct range were obtained when the standard was 40% C-hordein mixed with an inert protein. A preparative-scale method was developed to isolate and purify C-hordein, and C-hordein is proposed as a reference material to calibrate barley prolamin quantification in R5-based assays.

Key words: Prolamin, Gluten quantification, R5, Hordein, C-hordein, Gluten-free

Introduction

In order to predict the potential toxicity of cereal products for people with celiac disease or gluten sensitivity, gluten content is measured directly, since there is at present no assay that estimates all of the immunopathogenic elements. The currently most widely accepted method for gluten quantification is an enzyme-linked immunosorbent assay (ELISA) based on the R5 antibody. This is classified as a Type 1 method by Codex Alimentarius (standard 118-1979). With appropriate extraction, this method performs well in most food matrices when calibrated against wheat (*Triticum aestivum* L. emend Thell.) gliadin. In the quantification of hordein in barley-containing foods, however, the method leads to overestimation. Kanerva et al.[1] reported 7-30 times overestimation when barley (*Hordeum vulgare* L.) contamination in oat (*Avena sativa* L.) was measured with a gliadin reference material. When Hernando et al.[2] investigated the spiking of different barley cultivars in maize (*Zea mays* L.) flour, the R5 ELISA overestimated its content by 1.2 times. Later the same group[3] reported that overestimation of barley in maize flour was 1.8 times, when extracted with 'cocktail solution', a phosphate-buffered saline with reducing and disaggregating agents.[4] The Kd value, the equilibrium dissociation constant between the antibody and its antigen, of total hordein of cultivar Risø 56 against R5 antibody was 39 times greater than the that of the reference gliadin.[5] There is no data suggesting that barley has greater toxicity to coeliacs than wheat, so it is important to have correct gluten quantification of barley-containing products.

One reason for the overestimation may be that the composition of gliadin

reference material is substantially different from that of hordeins. The current gliadin reference material is a selection of 28 European wheat cultivars,[6] with 86.4% gliadin content, including 41.7% α-gliadin, 47% γ-gliadin, and 11.3% ω-gliadin by HPLC-RP analysis. When hordein is extracted with reducing agents, its typical composition is 7-8% D-hordein (corresponding to HMW-glutenin), 10-20% C-hordein (corresponding to ω-gliadin), 70-80% B-hordein (LMW-glutenins), and a minor amount of γ-hordein (corresponding to γ-gliadin).[7, 8, 9] Each of these protein groups has different reactivities against R5 antibody. The proportion of ω-gliadin, which is structurally similar to C-hordein, in total gliadin of common wheat and in the gliadin reference is less than the proportion of C-hordein in total hordein.

The R5 antibody recognizes mainly the epitope QQPFP, and homologous epitopes LQPFP, QLPYP, QQSFP, QQTFP, PQPFPF, QQPYP and PQPFP to a weaker degree.[10] C-hordein, the primary structure of which is almost entirely repeats of PQQPFPQQ, is strongly recognized by the R5 antibody and has 15-20 times more reactivity than the reference gliadin.[11] This explains the results of earlier barley overestimation.

Using a separate hordein reference material would make the quantification of barley prolamin more accurate, as previous studies have already shown.[1, 3, 5] The degree of overestimation ranges widely, because each research group has isolated its own hordein standard from different cultivars, so it is questionable whether a single whole hordein from one cultivar could be used to calibrate the hordein concentration in barley-containing products.

The aim of this study was to investigate the proportion of C-hordein in whole barley hordein, in order to explain the hordein overestimation with gliadin reference material in R5 antibody-based ELISA. An additional aim was to determine whether a reference material using C-hordein could be used to quantify hordein, for example, to determine the barley contamination in oats.

Materials and Methods

Materials

Twenty-nine barley cultivars for feed and malt purposes were selected for this study. Cultivars Brage, Tocada, Vilde, Marthe, Fairytale, Aukusti, Wolmari, Barke, Polartop, Einar, Jyvä, Saana, Edvin, NFC Tipple, Streif, Harbinger, Scarlett and Elmeri were kindly provided by Boreal Plant Breeding Ltd. (Finland). The sample of Xanadu was from the Kesko Corporation (Finland), and that of Propino from Syngenta (Finland). These were the top 20 cultivars grown in Finland during 2012-2015. In addition, cultivars KWS Asta, Lacey, Minttu, Toria, Overture, Voitto, Annabel and SW Mitja (Boreal Plant Breeding Ltd.) were analyzed. Hull-less cv. Jorma was from Risto Laitinen (Villala, Finland). The barley kernels were milled to flour with a Brabender Quadrumat Junior (Duisburg, Germany) and passed through an 850 μm sieve.

All the chemicals used were analytical quality or better. Bovine serum albumin (BSA) with purity ≥ 98% was purchased from Santa Cruz Biotechnology (Dallas, Texas).

Hordein composition by reverse-phase high-pressure liquid chromatography (RP-HPLC)

The hordein composition was determined by RP-HPLC. Barley flour (0.1 g) was extracted following the Osborne sequence of 1 ml of mQ-water, followed by 1 ml of 0.5 M NaCl, and 1 ml of mQ-water to remove the albumin and globulin proteins at ambient temperature. After each extraction, samples were centrifuged for 10 min at 20000 × *g*. The pellet was treated either once or 3 times with 40% (v/v) aqueous 1-propanol with 5% (v/v) 2-mercaptoethanol at 50°C for 30 min to extract the whole hordein fraction. The supernatants from the three sequential extractions were bulked together. Further samples of barley flour were also directly extracted either once or 3 times with the same solvent. After centrifugation, the supernatants from all four of these extraction types (Osborne or direct, single or triple) were filtered through a 0.45 µm membrane prior to RP-HPLC separation. An injection volume of 10 µl of whole hordein was separated on a C8 column (4.6 × 100 mm, 300 Å, 5 µm, Discovery, Sigma-Aldrich Co. LLC), at 35°C. A linear acetonitrile gradient from 20% solvent B (0.1% TFA in acetonitrile) to 50% solvent A (0.1% TFA in milli-Q water) was run over 40 min at a flow rate of 0.7 ml/min, and the elution was monitored at 210 nm. The hordein peaks were integrated (Empower 2, Waters Corporation) and the peak areas were compared to determine the C-hordein proportion of total hordein in each cultivar. Because the proportion of γ-hordein was reported to be very minor, and the HPLC profile did not distinguish it, it was counted together with B-hordein.

Hordein fractions reactivity against R5 antibody

The hordein fractions were collected, and their protein content was quantified with a BSA standard (linear range 0-60 µg, R^2 = 0.988) by peak area. The collected fractions were dried first under a nitrogen stream and then by vacuum centrifugation (Savant SpeedVac SC110A concentrator, USA). The dried fractions were completely solubilized in cocktail solution (R7006, R-Biopharm, Darmstadt, Germany) and analyzed by R5 sandwich ELISA (R7001, R-Biopharm, Darmstadt, Germany).

Purification of C-hordein in a preparative scale

The flour of cv. Jorma was first washed with acetone to remove phenolic compounds and filtered, then extracted following the Osborne sequence described earlier. The monomeric hordein was extracted with 40% (v/v) 1-propanol at 50°C without reducing agent. After centrifugation at 18000 × *g*, the supernatant was left at 4°C overnight. The suspension was centrifuged again to remove the precipitates before mixing with buffer A. Buffer A consisted of 40% (v/v) aqueous 1-propanol, 10 mM glycine, and 50 mM citric acid-sodium citrate buffer at pH 3.0, and buffer B consisted of 40% (v/v) aqueous 1-propanol, 0.5 M NaCl, 10 mM glycine, and 50 mM citric acid-sodium citrate buffer at pH 3.0. An ion exchange column (35 mm diameter × 300 mm length) was packed with TOYOPEARL SP-650M (TOSOH Bioscience LLC, Japan) and coupled with ÄKTAprime plus system (GE Healthcare, Sweden). After the sample was loaded, the separation gradient was 0-50% B from 0 ml to 600 ml at a flow rate of 15 ml/min. The eluate was collected in 10 ml fractions, and monitored at 280 nm. The components of fractions were analyzed by SDS-PAGE (NuPage Bis-Tris 10%, Invitrogen,

LifeTechnologies). The fractions containing C-hordein were combined and dialyzed against mQ-water with a cut-off MW 10 000 (Sigma-Aldrich D9527). The dialysed C-hordein was lyophilized (Heto-Holton DW8-85, Denmark). For the purpose of this study, this fraction was used as a benchmark of 'pure' C-hordein.

C-hordein as a reference material in sandwich and competitive ELISA

Lyophilized C-hordein and BSA were dissolved separately in 60% (v/v) aqueous ethanol at 0.3 mg/ml, and then mixed in ratios of 1:9 (C-hordein:BSA), 2:8, 3:7, 4:6 and 5:5, further referred as 10%, 20%, 30%, 40%, 50% C-hordein at a constant protein concentration of 0.3 mg/ml. Because BSA does not react with the R5 antibody, the mixture of C-hordein with BSA in different ratios was expected to mimic total hordein in ELISA. The samples were tested in sandwich and competitive ELISA assays (R7021, R-Biopharm, Darmstadt, Germany) to compare with the kit reference materials.

Prolamin quantification of oat flour spiked with barley flour

Flours of cultivars Elmeri, Einar and Marthe, with C-hordein contents of 33.1%, 25.6% and 17.4%, respectively, were selected for spiking in commercial gluten-free oat flour (Provena, Raisio Nutrition Ltd. Finland). An aliquot of 0.1 g of barley flour was added to 0.4 g whole grain oat flour and homogenized by vortexing. The mix was further diluted to 400 times by oat flour manually in steps (×4, ×10, ×10) to ensure homogeneity. The hordein concentration was also determined by R5 sandwich ELISA, and calculated with gliadin reference material and 40% C-hordein. The spiking was

conducted in three replicates and each replicate was measured in two dilutions, and the means and standard errors were calculated. The prolamin concentration of the three barley cultivars was also determined by RP-HPLC using BSA as standard by peak area. Flour was extracted with 40% (v/v) aqueous 1-propanol with 5% (v/v) 2-mercaptoethanol and injected on a C8 column as described earlier.

Results

Hordein HPLC profile and C-hordein proportion

When whole hordein was separated on a C8 column, D-hordein was eluted first, followed by C-hordein and B-hordein (Figure 1).[8] The 29 analyzed cultivars could be divided into three C-hordein peak patterns, of which type (b), for example cv. Barke, was the most common, present in 19 cultivars. The C-hordein proportion in total hordein of cv. Elmeri after a single extraction was 26.0% and after three sequenced extracts was 24.7%; the corresponding figures after Osborne extraction were 25.5% and 25.7%. There was no significant difference between the single and triple extraction results, so the C-hordein content of all cultivars was determined with a single direct extraction (Table 1).

From 2012 to 2015, cv. Barke and NFC Tipple were the two most popular malting cultivars in Finland, accounting for over 50% of the hectarage for this crop. Feed cultivars were far more diverse, with cv. Elmeri, Wolmari, Brage and Aukusti accounting for over 40% of the feed barley hectarage (National Land Survey of Finland, 2012-2015; http://www.maanmittauslaitos.fi/en). The C-hordein content of the 29 cultivars ranged two-fold, from 16.5% to 33.1% (Table 1). There was slight variation in the C-hordein content of cv. Elmeri from 2010, 2014 and 2015. According to this data set, the weighted average C-hordein content of whole hordein in Finland 2012-2015 was 25-26%.

Hordein subunit reactivity against R5 antibody

The reactivity of D-, C- and B-hordeins against the R5 antibody varied widely in sandwich ELISA (Figure 2). C-hordein was 10-20 times more reactive than gliadin standard, which in turn was 8-25 times more reactive than B-hordein. The slope of the curve indicated that C-hordein and gliadin standard had similar affinity with the R5 antibody, while B-hordein had less, and D-hordein had almost none. The three types of C-hordein and B-hordein reacted similarly with R5 antibody, although their HPLC patterns were different.

Preparation of C-hordein by FPLC

When hordein was extracted with 40% (v/v) aqueous 1-propanol without reducing agents and kept at 4°C overnight, precipitates appeared that were mainly composed of B-hordein. At pH 3, C-hordein and B-hordein were clearly separated in cation-exchange chromatography (Figure 3a). Fractions 11-15 (Peak 1) absorbed at 280 nm but did not contain prolamins, indicating that they were other compounds. Fractions 17-25 (Peak 2) contained C-hordein and were collected and combined in their native form. Following increased NaCl concentration, B-hordein eluted in fractions 54-57 (Peak 3) (0.08-0.11 M NaCl). HPLC of lyophilized C-hordein showed its high purity in comparison with the whole hordein (Figure 3b).

Purified C-hordein as reference material in ELISA

In sandwich ELISA, the affinity (the slope) of C-hordein with R5 antibody behaved similarly with gliadin standard, and at a ratio of 3 C-hordein: 7 BSA (30% C-hordein), the reaction almost matched that of the gliadin standard (Figure 4a). In competitive ELISA, the affinity of C-hordein with R5 antibody

was similar to that of the peptide standard, being closest at a ratio of 1 C-hordein: 9 BSA (10% C-hordein) (Figure 4b). Using the C-hordein and BSA in different ratios could replace both the gliadin standard in R5 sandwich ELISA, and the peptide standard in R5 competitive ELISA. In sandwich ELISA, the curves of purified whole hordein of common cultivars, such as cvs. Barke and NFC Tipple (C-hordein proportions 24.5% and 28.1% respectively), were above that of the gliadin standard and between that of the 30% and 50% C-hordein standard. The curve of cv. KWS Asta, with its low C-hordein content (16.5%), was close to the gliadin standard curve and that of 30% C-hordein (Figure 4c).

Measuring barley contamination in oat

With the same spiking, the determined prolamin concentrations of three barley flours were different by HPLC, R5 sandwich with gliadin standard, and R5 sandwich with 40% C-hordein standard, but in the same order of Elmeri > Einar > Marthe (Figure 5). The prolamin concentration calibrated by gliadin standard was 2.5 times (cv. Elmeri), 1.8 times (cv. Einar), and 1.2 times (cv. Marthe) the HPLC results. However, calibrated with the 40% C-hordein standard, the estimated prolamin concentration was 1.2 times (cv. Elmeri), 0.85 times (cv. Einar) and 0.63 times (cv. Marthe) the HPLC results. For cvs. Elmeri and Einar, the estimated value by the 40% C-hordein standard were not significantly different from those determined by HPLC, but for cv. Marthe the estimate was significantly lower, until the standard was changed to 30% C-hordein.

Discussion

This study investigated the reasons for the overestimation of barley prolamin when measured by the R5 antibody ELISA with the gliadin standard. C-hordein was 80-200 times more reactive with R5 antibody than B-hordein, whereas D-hordein did not react significantly with R5 antibody. When detecting whole hordein with R5, the main recognition was from C-hordein, so the proportion of C-hordein in whole hordein was crucial for correct prolamin quantification. The C-hordein proportion varied from 16.5% to 33.1% in common barley cultivars in Finland, with an average C-hordein around 26%. The composition of the gliadin standard was not comparable to that of hordein. Using the gliadin standard caused a 1.8-2.5 times overestimation of barley flour spiked in gluten-free oat flour, but use of a 40% C-hordein standard allowed calibration to give the correct concentration. A preparative-scale method was developed to isolate and purify C-hordein by cation-exchange chromatography, and C-hordein is proposed as a reference material to calibrate barley prolamin quantification in the R5 ELISA.

The varying reactivity of hordein subunits against the R5 antibody is attributable to the number of epitopes. The main R5 epitope, QQPFP, has been shown to occur 13 times in C-hordein (Uniprot Q40055), and minor epitopes QQPYP, QQTFP, PQPFP and QLPFP each occur once. One main QQPFP epitope and 7 minor epitopes were found in B3 hordein (Uniprot I6TEV5), and 5 QQPFP epitopes in B1 hordein (Uniprot P06470). Only one QQPFP epitope was found in λ3-hordein (Uniprot P80198) and no R5 epitope was found in D-hordein (Uniprot Q84LE9). Our results agreed with those of

Tanner et al.[5] who measured hordein subunits of cultivars Sloop and Risø 56 against R5 antibody and calculated their dissociation constant (Kd) value according to the best fit. In their study, the Kd value of B-hordein was 132 times than that of C-hordein in cv. Sloop, which means that it is much less sensitive to R5 antibody. In 'Risø 56', a B-hordein null cultivar, the Kd value of γ-hordein was 74 times greater than C-hordein. The Kd value of C-hordein differed two-fold in these two cultivars. The current study showed that the C-hordein of common European cultivars Harbinger, NFC Tipple and Barke behaved similarly in the recognition of the R5 antibody, although their peptide/protein composition as demonstrated by the HPLC profiles differed. C-hordein of a single cultivar can consist of up to 20 polypeptides, thus forming different HPLC profiles. The primary structures of these prolamin polypeptides are highly homologous, but their molecular weights differ.[12] In this study, three groups of cultivars were gathered according to the RP-HPLC profiles of their C-hordein, and an even more detailed classification is available based on the gel patterns.[13]

The ratio of B-hordein to C-hordein in this study ranged from 1.89 for cv. Elmeri to 4.68 for cv. KWS Asta. This range is comparable to that of Scottish cultivars, from 1.79 to 4.15, determined by a similar HPLC method.[14] The corresponding protein group to C-hordein in wheat is ω-gliadin, which shows about 70% sequence homology, with a similar repetitive sequence in the central domain of PFPQQPQQ.[15] The ω-gliadin content of total gliadin has been reported to range from 6.2% to 20.0%,[16] or from 10% to 19%.[17] High ω-gliadin proportions (17-20%) were found in feed wheat cultivars carrying

chromosome arm 1RS from rye.[16] The proportion of C-hordein in total hordein is generally higher than the proportion of ω-gliadin in total gliadin. The composition of prolamin is affected by cultivar, environment and nitrogen nutrition.[18, 19] In a set of barley cultivars grown in Spain and Scotland, the C-hordein proportion was highest in young endosperm, but at harvest ripeness, the B to C hordein ratio decreased slightly in Scotland while dropping much lower in Spain.[20] The C-hordein and ω-gliadin proportion was enhanced in low-sulphur growing conditions.[21, 22] The sulphur distribution varies in Europe, for example, in central and northern Germany, Poland, most of Sweden, and northern Finland, the subsoil sulphur value was lower than in northern Scotland, the Netherlands, north-eastern Germany and south-eastern Spain.[23] Increasing nitrogen fertilization increases both the hordein fraction of whole protein and the C-hordein proportion of whole hordein.[18] The proportion of ω-gliadin in total gliadin increases with nitrogen nutrition and temperature.[17, 24, 25]

A C-hordein standard could be used to determine the prolamin content in, for example, barley-contaminated oat, or purified barley starch, malt and malt extracts. Cross-contamination of oat can occur during growing, harvest, processing, storage and transportation, and the contamination of commercial oat should be no more than 4%. Barley is the most common contaminant in oat, since they are often part of the same cropping sequence.[2, 26] If the contaminant is suspected to be wheat, either a gliadin standard or 30% C-hordein could be used for calibration. If the contaminant is suspected to be barley, which is more likely, a C-hordein standard, based on the average C-

hordein proportion of the region, can be used for calibration. The process of barley starch extraction is under low pH so that acid hydrolysis of hordein may occur, hence competitive ELISA should be utilized to detect small fragments of prolamin, along with the sandwich assay. The prolamin of barley malt is partially hydrolyzed by its endogenous enzymes, so it should be quantified in both sandwich and competitive assays. In the sandwich assay, the current commercial reference material is the gliadin standard, while in the competitive assay it is a hydrolysate mixture of the gliadin standard, one barley cultivar and one rye cultivar.[27] Clearly, these two reference materials are not comparable. In addition, the commercial peptide standard was hydrolyzed by the digestive enzymes pepsin and trypsin, which is very different from the malting or the starch separation process. Our results showed that the 30% C-hordein and BSA mixture could represent gliadin standard, while the 10% C-hordein mixture can represent the peptide standard. Thus, the use of a C-hordein standard could not only allow correct determination of prolamin content, but also make the results from sandwich and competitive assays comparable.

Nevertheless, the validity of gliadin peptide standard for hydrolyzed products has not yet been confirmed. In quantification of prolamin in spiked beer by competitive R5 ELISA, calibration using gliadin peptide standard resulted in overestimation by 180%.[28] The difficulty of prolamin quantification in beer is that the raw material, type and level of hydrolysis vary widely among products. The peptides in the standard may not be representative of the prolamin hydrolysates in beer. Competitive R5 detects peptides longer than five amino

365 acids, but small peptides with 5-9 amino acids are considered non-toxic to
366 coeliac patients.[29] New methods are under development to detect prolamin
367 peptides in beer, such as LC-MS/MS,[30, 31, 32, 33] and an ELISA method based
368 on the G12 antibody.[34] Further investigations should include comparison of
369 the R5 reactivity of hordein with that of hordein peptides hydrolyzed by
370 different enzymes, and how C-hordein as reference material can represent
371 the hydrolysates in hydrolyzed products.
372
373 In analytical work, a reference material or standard is necessary. For R5-
374 based gluten analysis, the PWG gliadin standard is available, but it is not
375 reproducible, and it is not accepted as a certified reference material in the
376 Institute of Reference Material and Measurements of the European
377 Commission due to its high glutenin content (Working Group on Prolamin
378 Analysis and Toxicity, 2016; http://www.wgpat.com/pwggliadin.html).
379 Validated and certified reference material is still missing in Codex 118-1979
380 standard. C-hordein as reference material is a group of homologues of
381 proteins, rather than a mixture of gliadins from different wheat cultivars. C-
382 hordein is a better substrate for R5 antibody because its repetitive sequences
383 correspond to R5 epitopes. Additionally, the use of C-hordein as calibrant has
384 the possibility to adjust by the percentage of C-hordein. Other antibody-based
385 (G12, Skerritt, α-20) methods have their own reference materials.[34, 35, 36]
386 Researchers are trying to develop wheat-based reference materials
387 applicable to specific food products.[37, 38]
388

In conclusion, this study determined that the high proportion of C-hordein in total hordein is the reason for the consistent overestimation of hordein by the R5 ELISA assay which uses gliadin as reference material in gluten-free analysis. We isolated C-hordein and propose it as the reference material for quantifying hordein concentration in barley-containing foods, including those that may have been contaminated with barley.

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Figure 1. Reverse-phase liquid chromatogram of total hordein extract separated on a C8 column. (a) cv. Harbinger; (b) cv. Barke; (c) cv. NFC Tipple. The total hordein was directly extracted with 40% (v/v) 1-propanol with 5% (v/v) 2-mercaptoethanol from barley flour.

Figure 2 Reaction of isolated hordeins against R5 antibody in sandwich ELISA. Three types of C-hordein and B-hordein were from cultivars Harbinger, Barke and NFC Tipple. D-hordein was from cv. Harbinger.

Figure 3 (a) Cation-exchange chromatogram of hordein. The black line shows the absorbance at 280 nm, fractions from peaks 1, 2 and 3 were collected, and the red line shows the gradient concentration of NaCl. The inset shows SDS-PAGE of collected fractions; (b) Reverse-phase chromatogram of purified C-hordein and whole hordein from cv. Jorma.

Figure 4 Reaction of C-hordein mixed with bovine serum albumin (BSA) in different ratios against R5 antibody (a) in sandwich ELISA; and (b) in competitive ELISA. (c) Reaction of purified whole hordein of 6 cultivars in R5 sandwich ELISA compared with 30%, 40% and 50% C-hordein standards and gliadin standard.

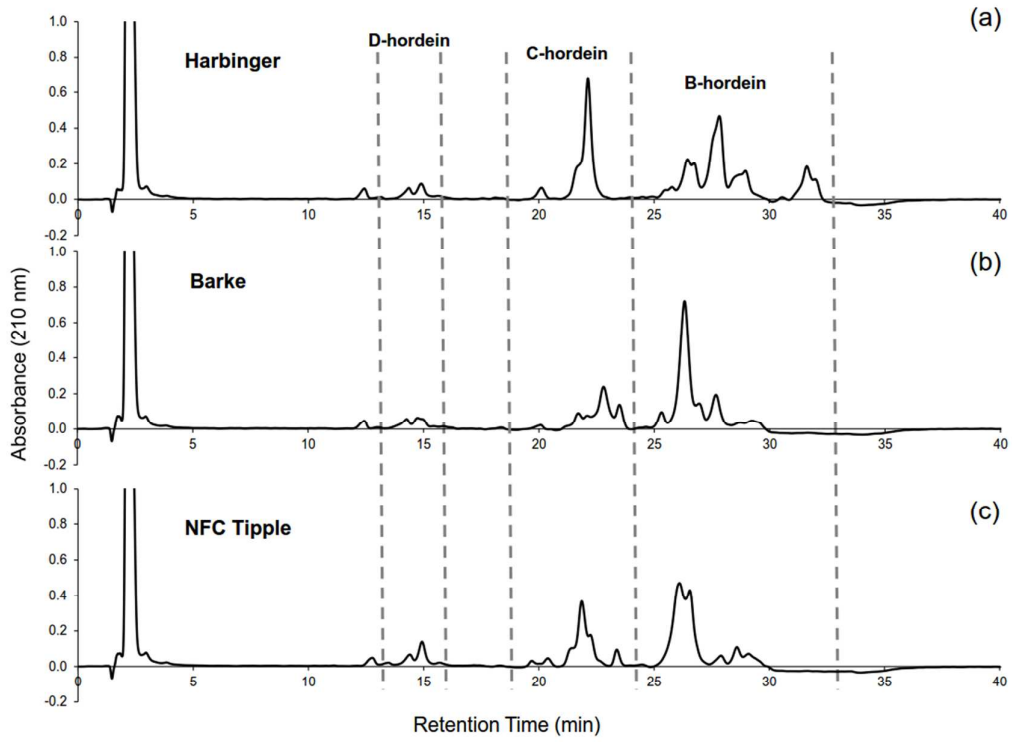
Figure 5 Prolamin concentration of gluten-free oat flour spiked with three barley flours, determined by HPLC, R5 sandwich ELISA with 40% C-hordein standard, and R5 sandwich ELISA with gliadin standard. Error bars show standard error.

570 **Table 1** C-hordein content as percentage of whole hordein of selected barley
571 cultivars.

Cultivar	Year	Origin	Content (%)	Cultivar	Year	Origin	Content (%)
KWS Asta	2013	CE	16.5	Saana	2014	Nordic	27.5
Marthe	2009	CE	17.4	Edvin	2010	Nordic	28.0
Tocada	2013	CE	18.6	Elmeri	2015	Nordic	28.1
Vilde	2010	Nordic+CE	18.6	NFC Tipple	2013	CE	28.1
Propino	2014	UK	20.3	Brage	2010	Nordic	28.3
Fairytale	2013	CE	20.7	Streif	2014	CE	28.5
Toria	2010	Nordic	22.6	Minttu	2013	Nordic	28.8
Overture	2013	CE	23.0	Elmeri	2014	Nordic	29.2
Aukusti	2010	Nordic	23.1	Harbinger	2014	Nordic	29.6
Wolmari	2010	Nordic	23.3	Scarlett	2013	CE	30.0
Barke	2013	CE	24.5	Jorma	2011	Nordic	30.5
Polartop	2010	Nordic	24.5	Voitto	2010	Nordic	31.1
Einar	2010	Nordic	25.6	Xanadu	2014	CE	31.1
Jyvä	2013	Nordic	25.6	Lacey	2010	NA	32.8
SW Mitja	2014	Nordic	25.7	Elmeri	2010	Nordic	33.1
Annabel	20	CE	25.9				

572 CE = central Europe
573 NA = North America
574

575 Figure 1



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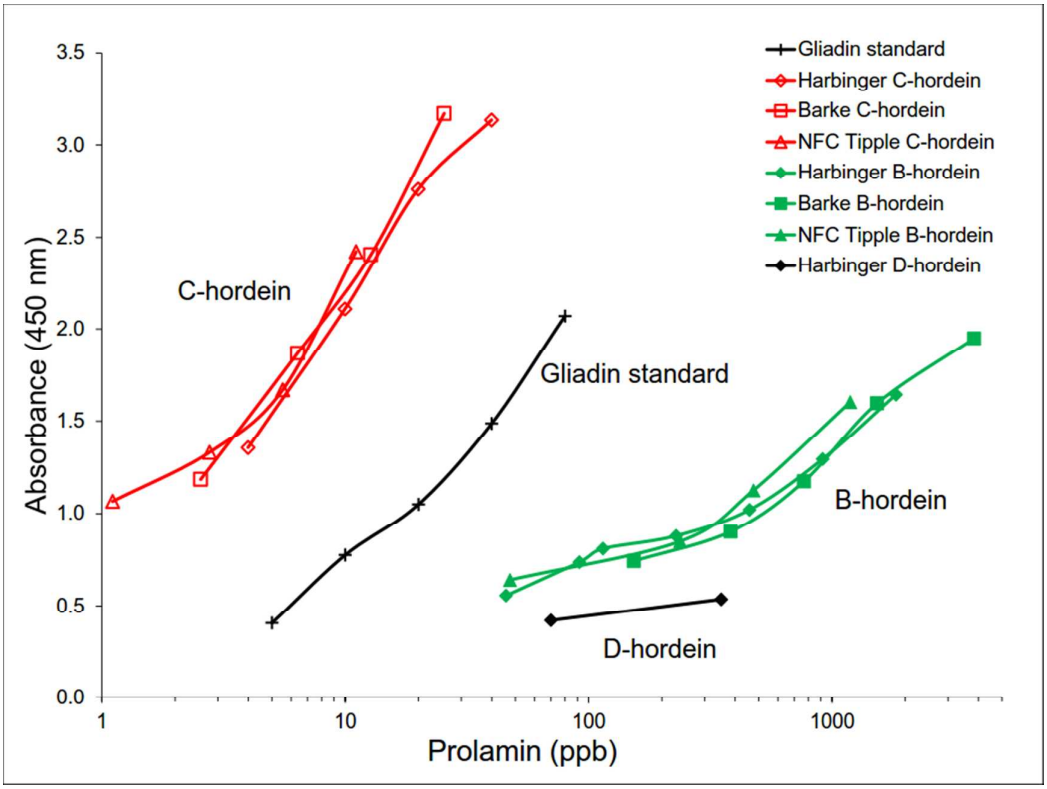
578 Figure 2



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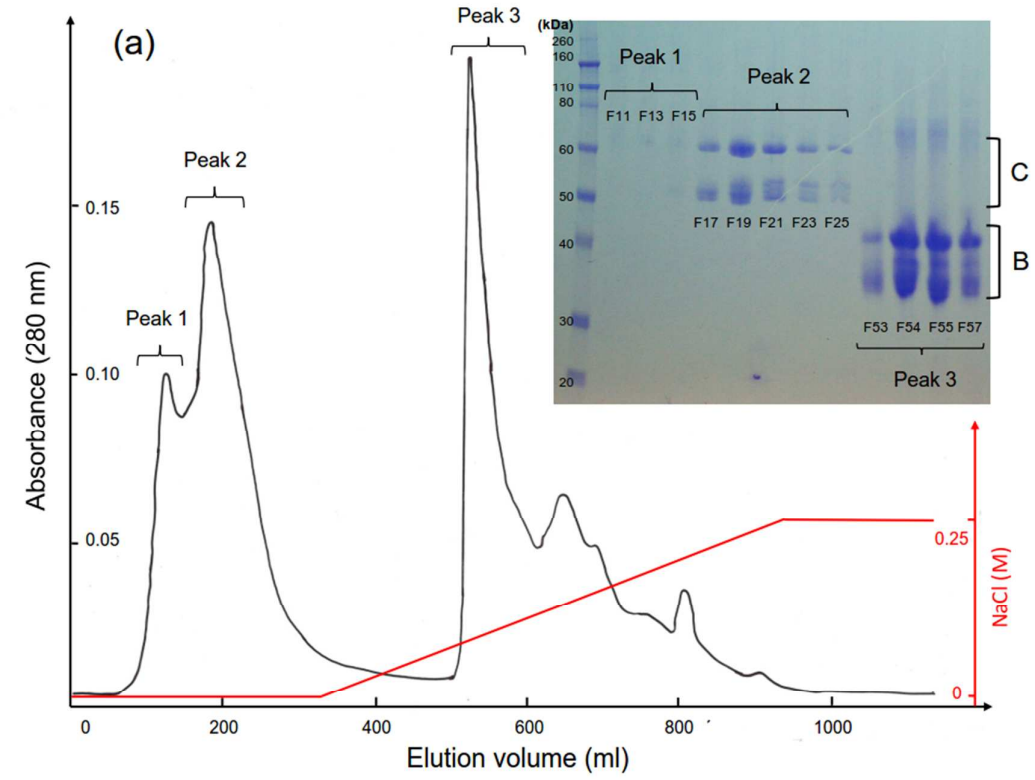
581 Figure 3



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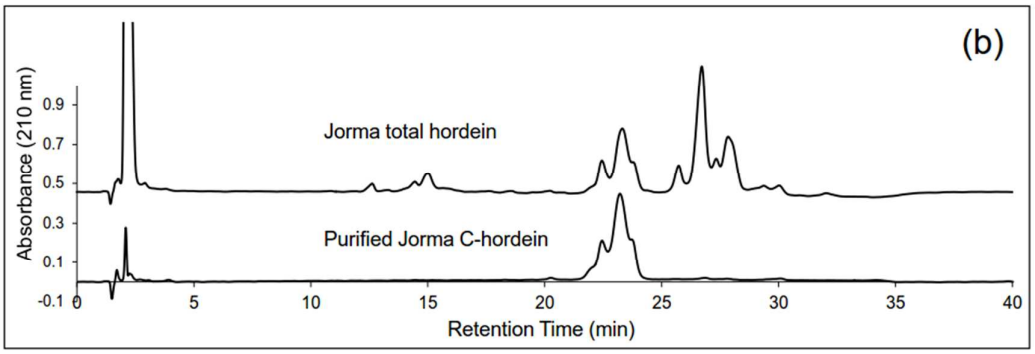

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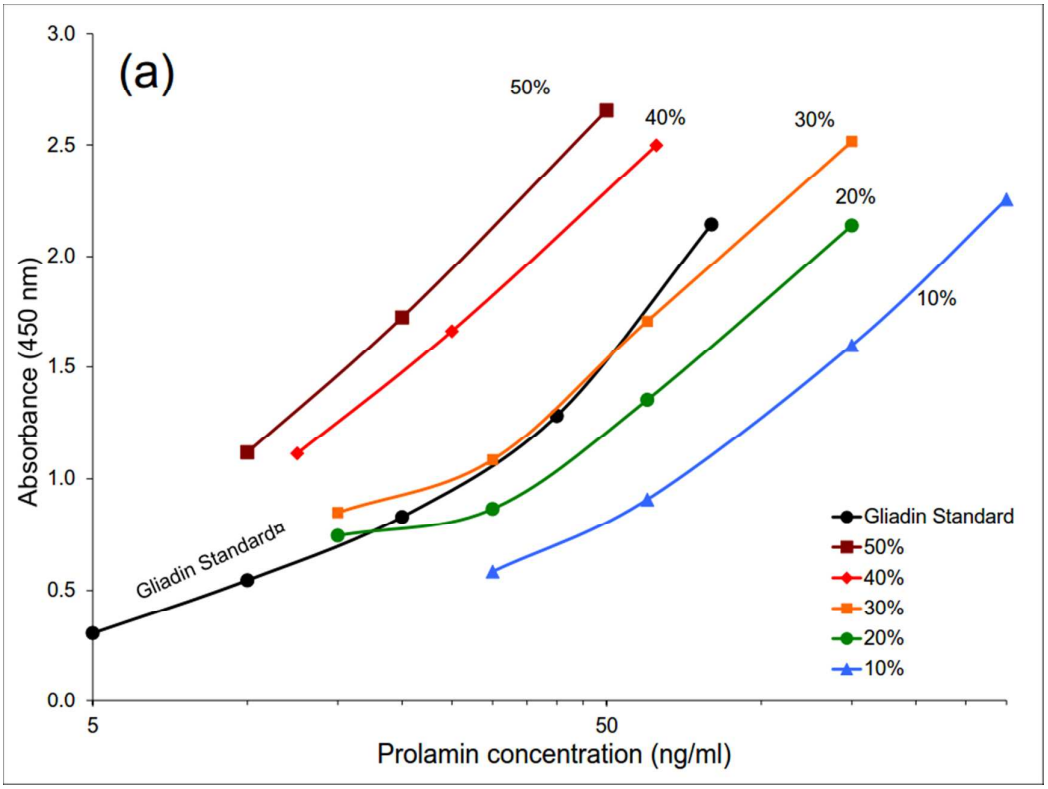

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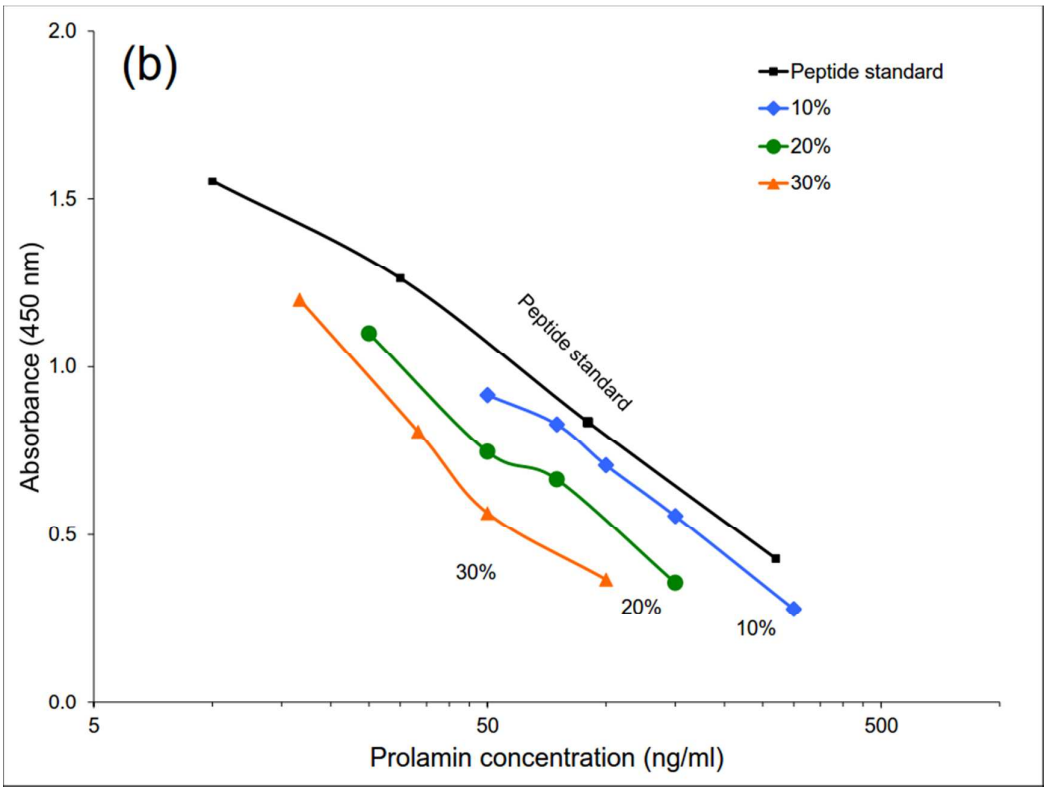

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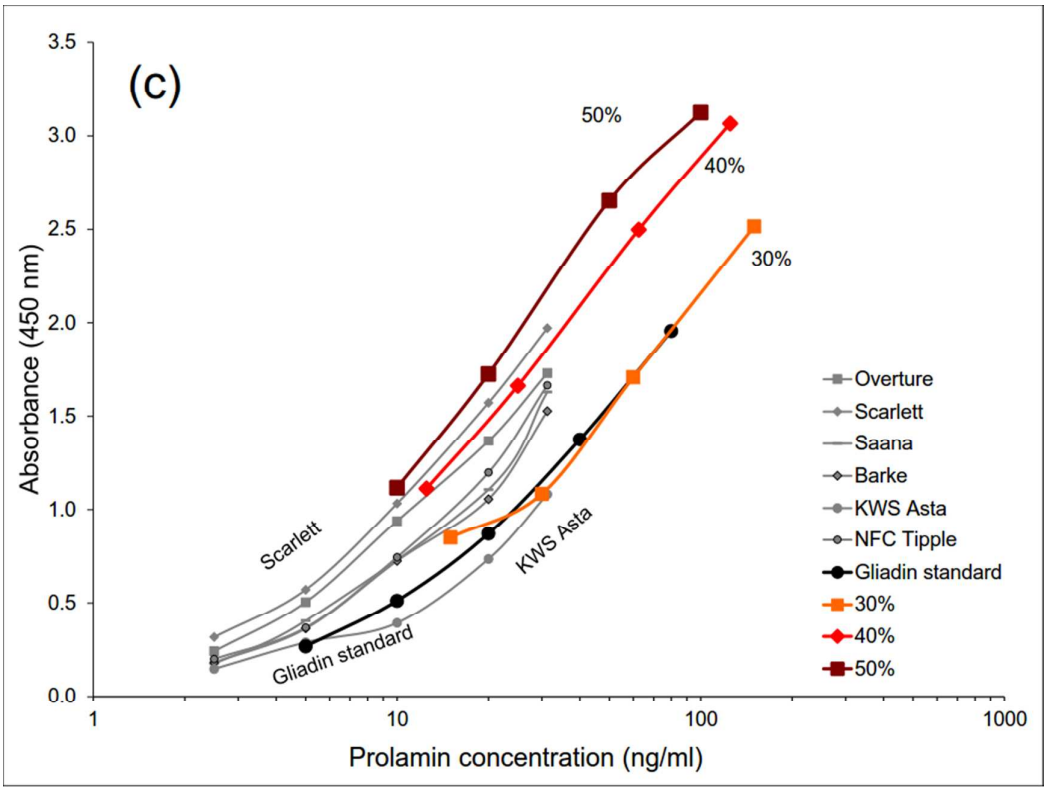
(c)
Absorbance (450 nm)
Prolamin concentration (ng/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
10
100
1000
50%
40%
30%
Scarlett
KWS Asta
Gliadin standard
Overture
Scarlett
Saana
Barke
KWS Asta
NFC Tipple
Gliadin standard
30%
40%
50%

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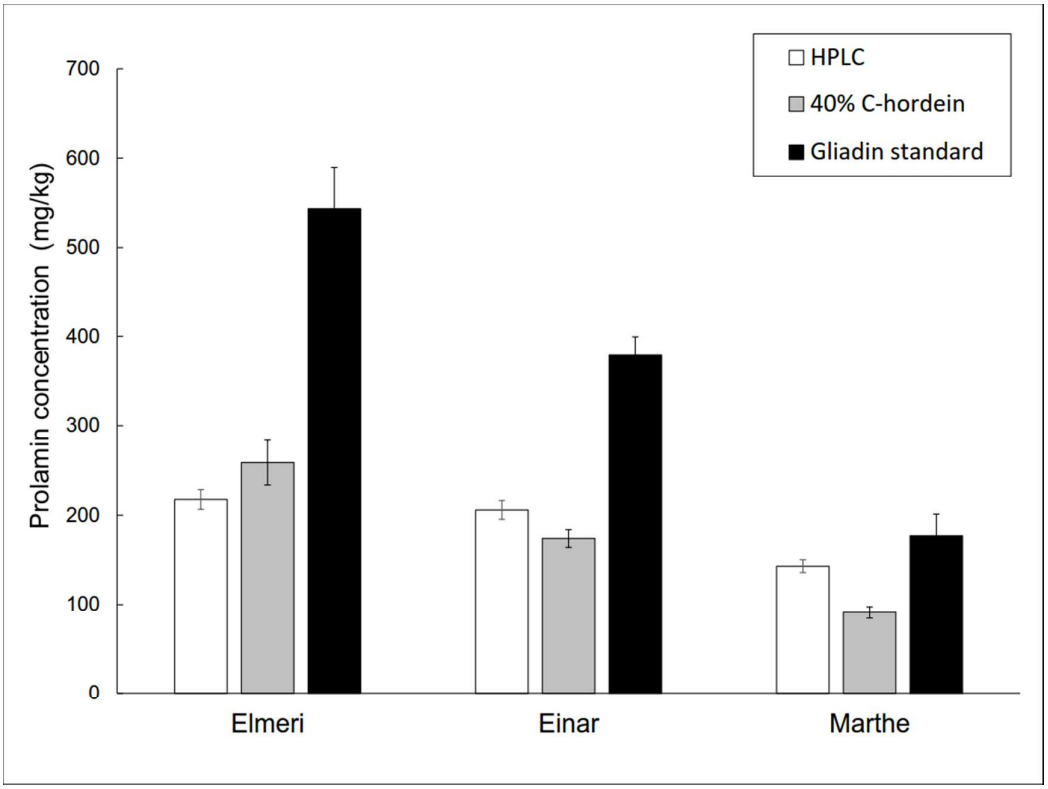
590 Figure 5



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